

Mechanistic Studies of Inactivation of Inducible Nitric Oxide Synthase by Amidines

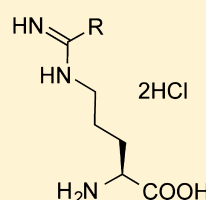
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S Supporting Information

ABSTRACT: Nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to L-citrulline and nitric oxide. *N*⁵-(1-Iminoethyl)-L-ornithine (L-NIO), an amidine-containing molecule, is a natural product known to be an inactivator of inducible NOS (iNOS). Because of the presence of the amidine methyl group in place of the guanidine amino group of substrate L-arginine, the active site heme peroxy intermediate sometimes cannot be protonated, thereby preventing its conversion to the heme oxo intermediate; instead, a heme oxygenase-type mechanism occurs, leading to conversion of the heme to biliverdin. This might be a new and general inactivation mechanism for heme-containing enzymes. In the studies described here, we attempted to provide support for amidines as substrates and inactivators of iNOS by the design and synthesis of amidine analogues of L-NIO having groups other than the amidine methyl group. No nitric oxide- or enzyme-catalyzed products could be detected by incubation of these amidines with iNOS. Although none of the L-NIO analogues acted as substrates, they all inhibited iNOS; increased inhibitory potency correlated with decreased substituent size. Computer modeling and molecular dynamics simulations were run on **10** and **11** to rationalize why these compounds do not act as substrates. Unlike the methyl amidine (L-NIO), the other alkyl groups block binding of O₂ at the heme iron. Compounds **8**, **9**, and **11** were inactivators; however, no heme was lost, and no biliverdin was formed. No kinetic isotope effect on inactivation was observed with perdeuterated ethyl **8**. A small amount of dimer disruption occurred with these inactivators, although the amount would not account for complete enzyme inactivation. The L-NIO analogues inactivate iNOS by a yet unknown mechanism; however, it is different from that of L-NIO, and the inactivation mechanism previously reported for L-NIO appears to be unique to methyl amidines.



- 4**, R = CH₃, L-NIO
7, R = H
8, R = CH₂CH₃
9, R = CH₂CH₂CH₃
10, R = CH(CH₃)₂
11, R = CH₂CH(CH₃)₂
12, R = C(CH₃)₃
13, R = Bn

Nitric oxide (NO) is an important cell-signaling agent, which is synthesized *in vivo* from oxygen and L-arginine by the enzyme nitric oxide synthase (NOS, EC 1.14.13.39).¹ The importance of understanding the chemistry and biochemistry of NO has been highlighted in fields as diverse as immunology and reproductive technology, while the inhibition of NO biosynthesis has been an important approach for the design of drugs for the treatment of septic shock, rheumatoid arthritis, stroke, Alzheimer's disease, and Parkinson's disease.^{2–5} There are three mammalian NOS isoforms, neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS); their sequences are 50–60% identical, and the enzymes share an identical overall architecture.^{6,7} The active sites of NOS isozymes are highly conserved; 16 of 18 residues within 6 Å of the substrate binding site of nNOS and eNOS are identical, and the side chain of one of the two dissimilar amino acids points out of the substrate binding site.⁸ Therefore, there is only a one-amino acid difference of which to take advantage in the active sites of these two isoforms.

However, because NOS inhibition may also be detrimental to the essential functions of NO, selective inhibition of individual

NOS isoforms is important for the development of therapeutics.

In the presence of O₂, NOS converts L-arginine (**1**) to L-citrulline (**3**) and NO with concomitant oxidation of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) (Scheme 1). Numerous cofactors bind to NOS, including flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), NADPH, protoporphyrin IX, and tetrahydrobiopterin (H₄B). Each NOS isoform is dependent on calmodulin (CaM), although iNOS has been shown to bind CaM very tightly regardless of the change in cellular Ca²⁺ levels;⁹ therefore, iNOS is kinetically independent of Ca²⁺ concentration. NOS is active only as a homodimer,¹⁰ with each monomer consisting of two subdomains. Inspection of the peptide sequence of NOS and the crystal structure of iNOS¹¹ reveals a C-terminal domain and an N-terminal domain. Sequences in the C-terminal domain of NOS contain binding sites for FMN, FAD, and NADPH.¹² The C-terminal domain serves to shuttle electrons from the two-electron donor,

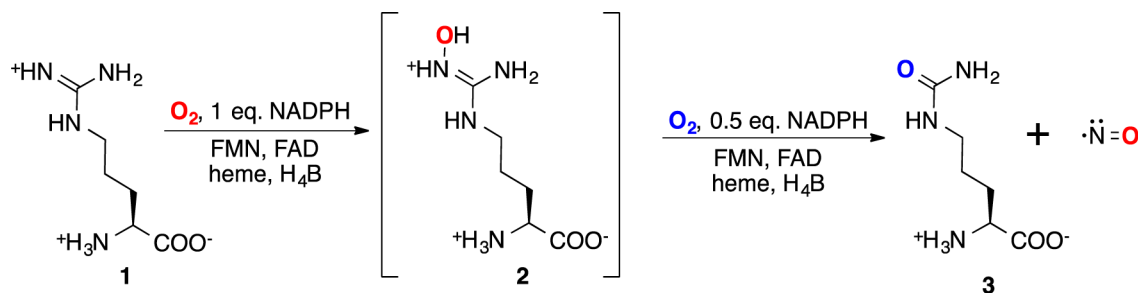
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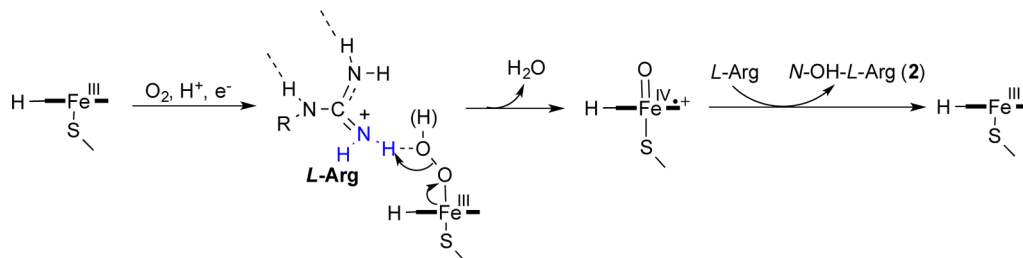
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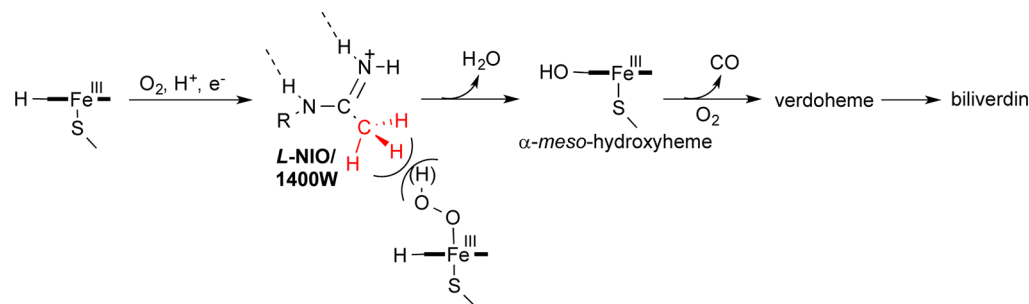
Scheme 1. Nitric Oxide Synthase-Catalyzed Conversion of L-Arginine (1) to L-Citrulline (3) and NO



Scheme 2. General Mechanism for the First Step of the NOS Reaction



Scheme 3. Proposed Mechanism of L-NIO and 1400W Inactivation with iNOS²⁶



NADPH, through the one- or two-electron acceptors/donors, FMN and FAD, to the H₄B^{13,14} and heme cofactors in the N-terminal domain of the other monomer.¹⁵ The H₄B is believed to be involved in the donation of an electron to the heme,^{16–18} where substrate oxidation occurs;^{19,20} during the catalytic cycle, the oxidized H₄B becomes reduced.^{21,22} Calmodulin and Ca²⁺ are required to allow the electron transfer between the domains.^{23,24}

Fast et al.²⁵ reported that the natural product N⁵-(1-iminoethyl)-L-ornithine (L-NIO) (4) is a time-, concentration-, and NADPH-dependent iNOS inactivator, and it acted as a substrate as well as an inactivator of iNOS. Incubation of iNOS with L-NIO leads to loss of the heme cofactor from the enzyme and the formation of biliverdin, as monitored by high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS) analysis. The same conversion of heme cofactor to biliverdin was identified by Zhu et al.²⁶ from the inactivation of iNOS by N-[3-(aminomethyl)benzyl]acetamide (1400W) (5). It was proposed that L-NIO, 1400W, and possibly all amidine-containing inactivators of NOS inactivate the enzyme by preventing protonation of the heme peroxide intermediate, which is believed to occur by the guanidinium group of L-arginine, thereby preventing the formation of the heme iron–oxo species and the normal substrate oxygenation (Scheme 2). Instead, a self-oxidation and irreversible inactivation of NOS

occur with conversion of its heme cofactor into biliverdin, along with the production of carbon monoxide (Scheme 3). This is a unique example of inactivation of an enzyme, because the inactivator is not modified during the inactivation phase. On the basis of the results that L-NIO acts both as a substrate and as an inactivator, we hypothesized that L-NIO might bind with either the CH₃ or the NH₂⁺ adjacent to the catalytic heme; when the NH₂⁺ group is rotated over the heme, oxygenation to N-hydroxy-L-arginine occurs [2 (Scheme 1)], but when the methyl group is rotated toward the heme, inactivation occurs (Scheme 3). This is a reasonable hypothesis because interchange of the CH₃ and the NH₂⁺ groups in L-NIO would not cause any steric problems in the NOS active site. However, it is known that N^ω,N^ω-dimethylarginine (6a) is an endogenous NOS inhibitor, but N^ω,N^ω-dimethylarginine (6b) is inactive,²⁷ indicating that the binding of 6b is intolerable in the NOS pocket, which is too narrow to allow the methyl group on both terminal guanidine nitrogens. We, therefore, designed and synthesized analogues of L-NIO with variable groups (R) at the terminus (Figure 1) to determine whether rotation of the amidine bond is indeed affected by R group size, and if it determines substrate turnover compared with inactivation. The results of these studies are described here.

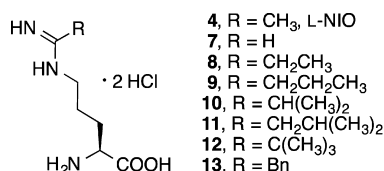
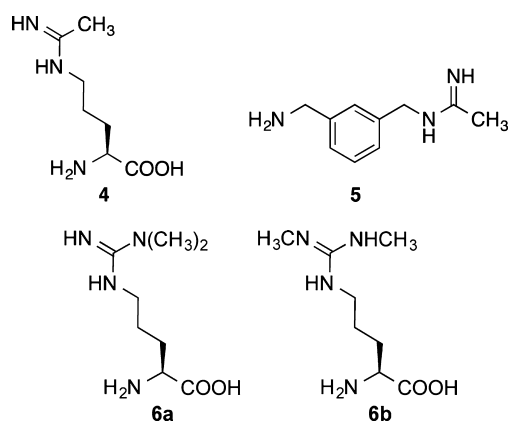


Figure 1. Analogues of L-NIO.



EXPERIMENTAL PROCEDURES

General Methods. All syntheses were conducted under anhydrous conditions in an atmosphere of argon, using flame-dried apparatus and employing standard techniques in handling air-sensitive materials. Nonsynthesized reagents were purchased from Sigma-Aldrich Co. and TCI and were used without further purification. Propanenitrile-*d*₃ was purchased from CDN Isotopes. ¹H NMR spectra were recorded on 500 MHz Varian or Bruker AVANCE spectrometers. Data are presented as follows: chemical shift (in parts per minute on the δ scale relative to δ 0.00 for the protons in TMS), integration, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), and coupling constants (*J* in hertz). Coupling constants were taken directly from the spectra and are uncorrected. ¹³C NMR spectra were recorded at 125 MHz, and all chemical shift values are reported in parts per million on the δ scale. All ion exchange chromatography was performed using Dowex 50WX8-200 resin. Chemical shifts are reported as δ values in parts per million with the CDCl₃, D₂O, and CD₃OD peaks set at 7.26, 4.80, and 3.31 ppm, respectively. An Orion research model 701H pH meter with a general combination electrode was used for pH measurements. LC–MS (ESI) was conducted on an Agilent MSD mass spectrometer. High-resolution mass spectra (HRMS) were measured with an Agilent 6210 LC-TOF (ESI) mass spectrometer. Optical rotations were measured on a PerkinElmer model 341 digital readout polarimeter. The enzyme assay was monitored on a BioTek Synergy 4 microplate reader. A Beckman System Gold 125P solvent module was used to conduct HPLC.

Enzyme Expression and Purification. The expression and purification of recombinant murine iNOS from *Escherichia coli* were performed according to the methods reported by Stuehr and Ikeda-Saito.²⁸

Hemoglobin Assay for iNOS Activity. The production of NO was measured by the rapid oxidation of oxyHb to metHb by nitric oxide.²⁸ The assay mixture contained L-arginine (10 μ M), NADPH (100 μ M), oxyhemoglobin (0.125 mg/mL), tetrahydrobiopterin (10 μ M), and different amounts of inhibitors. The final volume was adjusted to 600 μ L with 100

mM Hepes buffer (pH 7.4). The enzymatic reaction was initiated by addition of 10 μ L of an iNOS stock, and the rate of NO production was monitored by the change in absorbance at 401 nm in the initial 60 s on a spectrophotometer at 37 °C. Curves were fit using the Michaelis–Menten equation in GraphPad Prism 5.0 (GraphPad Software, Inc.). For *K*_i determinations, IC₅₀ values were calculated using nonlinear regressions (dose–response inhibition, four-parameter variable slope). Subsequent *K*_i values were calculated using the Cheng–Prusoff relationship: *K*_i = IC₅₀/(1 + [S]/*K*_m) (*K*_m for murine iNOS is 8.3 μ M).²⁹

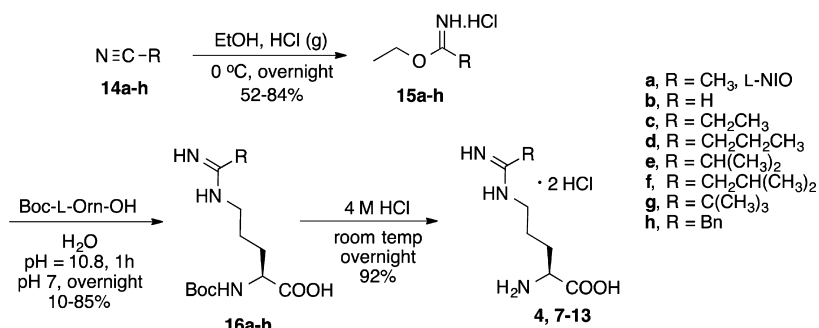
HPLC of Amino Acid Metabolites. The inactivation mixture was derivatized with *o*-phthalaldehyde and 2-mercaptoethanol. Inactivation mixtures contained 11.2 μ L of 4, 7–13 (2 mM), 6.7 μ L of Hepes buffer (100 mM, pH 7.4), 3.3 μ L of catalase (3.42 mg/249 μ L), 13.8 μ L of glycerol, 32 μ L of NADPH (39 mM), 15.7 μ L of H₄B (8 mM), and 64 μ L of an iNOS stock. The mixture was incubated at 37 °C for 3 h until inactivation had reached completion. Aliquots (10 μ L) were removed and added to 20 μ L of the *o*-phthalaldehyde/2-mercaptoethanol [20:1 (v/v)] reagent. The sample was injected onto an Econosil C₁₈ HPLC column (Alltech, 10 μ m, 250 mm \times 4.6 mm). A Beckman System Gold 125P solvent module was used to control the gradient elution as follows: isocratic elution with 90% solvent A and 10% solvent B conducted for 5 min. The level of solvent B was then increased linearly to 100% over a period of 10 min; 100% solvent B was employed for 10 min. Solvent A was 50 mM sodium acetate (pH 5.9). Solvent B consisted of 80% (v/v) methanol and 20% (v/v) solvent A. Sample elution was detected by absorbance at 340 nm with a flow rate of 1.0 mL/min.

Irreversible Inhibition Kinetics. The same inactivation mixtures were prepared as noted in HPLC of Amino Acid Metabolites. The reactions were initiated by the addition of enzyme, and 10 μ L aliquots were removed at specified time points and tested via the initial velocity assay. Controls were performed by replacing the inhibitor solution with Hepes buffer. *K*_i and *k*_{inact} values were determined by the method of Kitz and Wilson.³⁰

Activity Recovery Determination. The incubation mixture for enzyme activity recovery determination contains 70 μ M potential inactivator. When the incubation mixture showed an obvious activity loss after 16 h at 0 °C, microdialysis was performed over 16 h. The total incubation volume was 100 μ L, which was dialyzed in a volume of 100 mL of Hepes buffer (pH 7.4). The Hepes buffer was changed two times, every 5 h. All of the incubation mixtures with different compounds and the control were distributed into separate flasks to prevent any cross-contamination. The enzyme activity recovery was checked after overnight dialysis.

HPLC and LC–MS Determination of Biliverdin. Biliverdin determination after treatment of iNOS with 4 and 7–13 was conducted via LC–MS with diode array detection, using a C₁₈ reversed-phase column (Vydac, 218TP54, 5 μ m, 4.6 mm \times 250 mm) at 401 nm with 60% H₂O (0.1% TFA) and 40% CH₃CN (0.1% TFA) at a flow rate of 1.0 mL/min. Injections were made after the incubation mixture containing compound 4, 8, 9, or 11 no longer showed iNOS activity. A gradient method was used: solvent B began at 40% and was then increased linearly to 60% over a period of 10 min; 60% solvent B was decreased to 40% solvent B over 2 min, and 40% solvent B was employed for 3 min.

Scheme 4. Synthesis of Compounds 4 and 7–13



Computer Modeling of 10 and 11 in iNOS. Models of 10 and 11 were manually built according to the binding model of substrate (L-Arg) and docked to the murine iNOS structure (IN2N). Crystallographic waters were retained, and the system was further solvated in an octahedral unit cell with a 10 Å cushion. Sodium ions were added to maintain net neutrality giving a total of 75195 and 75201 atoms for the 10 and 11 complexes, respectively. The Amber 12.0 suite (<http://ambermd.org/>) was used for all calculations.³¹ The ff10 force field provided with the Amber 12.0 package was used for the protein, while the heme-Cys ligand parameters were taken from ref 32. Parameters for 10 and 11 were derived with antechamber and the gaff force field³³ using the BCC charging scheme.^{34,35}

The structure was prepared for production molecular dynamics runs by first energy minimization for 1000 cycles with all heavy atoms except water molecules fixed in position followed by another 1000 cycles where only the inhibitor atoms were allowed to move. In the final 1000 cycles, all atoms were allowed to move. Production runs were conducted with a 1 fs time step and coordinates saved every 10 ps. The temperature and pressure were held constant through weak coupling with a 1 ps pressure relaxation time and Langevin dynamics using a collision frequency of 1 ps⁻¹. Periodic boundary conditions were used with a particle mesh Ewald implementation of the Ewald sum for the description of long-range electrostatic interactions.³⁶ A spherical cutoff of 10.0 Å was used for nonbonded interactions. Bonds involving hydrogen atoms were constrained using SETTLE.³⁷ Analysis was conducted using the Amber software suite.

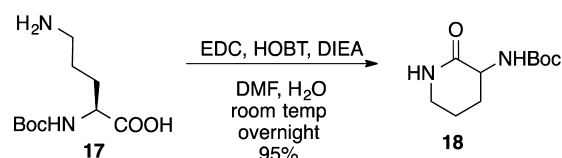
RESULTS

Syntheses of 4, 7–13, 22, and 26. Compounds 4 and 7–13 (Figure 1) were synthesized as shown in Scheme 4. The synthesis of all of the L-NIO analogues started by alcoholysis of nitriles 14, converting them to the corresponding imidic esters (15).^{25,38,39} Boc-protected L-NIO analogues (16) were produced by condensation of Boc-L-Orn-OH and 15. After removal of the Boc group by treatment with 4 M aqueous HCl, compounds 4 and 7–13 were obtained.

The reactions producing 10–12 did not proceed to completion, despite extended reaction times, and the unreacted Boc-L-Orn-OH was difficult to remove by chromatography. Therefore, to remove 17, the reaction mixture was subjected to *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide (EDC) lactamization (Scheme 5),⁴⁰ to form lactam 18, which was easily removed by extraction.

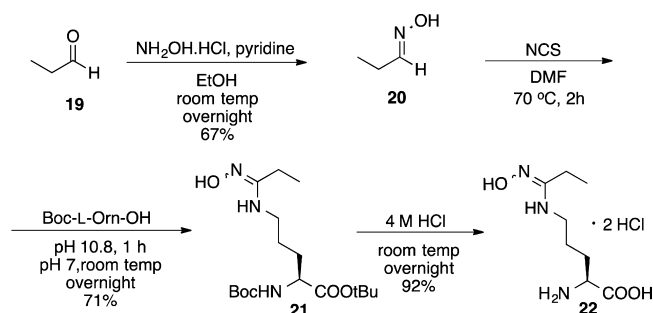
Compound 22, the potential hydroxylation metabolite of 8, was synthesized as shown in Scheme 6; oxime 20 was made

Scheme 5. Method for Removing 17 from the Reaction Mixture



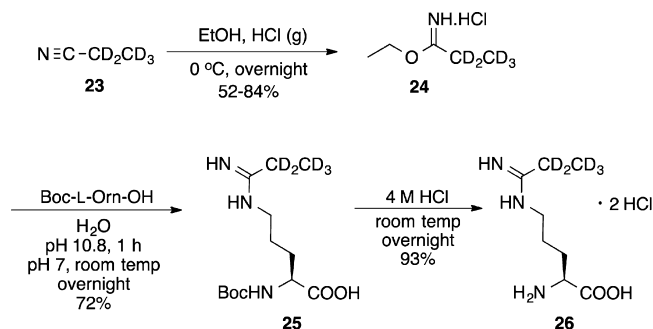
from propanal (19).⁴¹ Compound 22 was obtained after removal of the Boc protecting group.

Scheme 6. Synthesis of Compound 22



Compound 26 was successfully prepared following the synthetic route shown in Scheme 7. Starting with propane-

Scheme 7. Synthesis of 26



nitrile-*d*₅ (23), a Pinner reaction was used to make imidic ester 24. Substitution with Boc-L-Orn-OH gave 25, and the Boc group was removed in 4 M HCl to give 26.

Determination of Substrate Activity, Inhibition Constants (IC₅₀), and Inhibition Type. None of the L-NIO analogues generated any NO at concentrations up to 2 mM. Inhibition experiments were performed by measuring the

velocity of NO generation after using different concentrations of the inhibitor and different concentrations of substrate L-arginine. Inhibition of iNOS decreased as the alkyl group became more bulky (Table 1). The only exception is the reversal of K_i values for **10** and **11**. L-NIO and all analogues except **12** were found to be competitive inhibitors; **12** was a noncompetitive inhibitor.

Table 1. IC_{50} and K_i Values for Compounds **4** and **7–13** against iNOS

compound	IC_{50} (μM) ^a	K_i (μM) ^b
4 (L-NIO)	8.1	3.65
7	0.4	0.18
8	16.1	7.25
9	19.1	8.61
10	138	65.18
11	68.7	30.95
12	323	146
13	5333	2402

^aThe apparent IC_{50} values are represented as the mean from two or more independent experiments performed in duplicate with five or six data points each and correlation coefficients of 0.92–0.99. The experimental standard deviations were less than 10%. ^bThe apparent K_i values were obtained by measuring the percent enzyme inhibition in the presence of 10 μM L-arginine with at least five inhibitor concentrations. The parameters of the following inhibition equation⁴² were fit to the initial velocity data: % inhibition = $100[I]/[I] + K_i(1 + [S]/K_m)$. The K_m value for L-arginine was 8.2 μM (iNOS).

Inactivation of iNOS by **8, **9**, and **11**.** Compounds **8**, **9**, and **11** (Figure 1) were inactivators of iNOS, and their dissociation constants were measured.⁴³ Inactivation experiments were performed by measuring the enzyme activity remaining after incubation with different concentrations of the inactivator with the cofactors and iNOS. As shown in Table 2, L-NIO (**4**) is a more efficient inactivator than **8**, **9**, and **11**, although the inactivation rate constants (k_{inact}) for **8**, **9**, and **11** are greater than that for **4**.

Metabolites from Inactivation of iNOS by **8, **9**, and **11**.** HPLC analysis of the amino acid metabolites from **8**, **9**, and **11** inactivation using *o*-phthalaldehyde (OPA) and 2-mercaptoethanol (MCE) was conducted.^{25,44,45} LC–MS was employed to characterize any metabolites formed; however, none was detected. Compound **22** was synthesized as a standard for the potential N-hydroxylated metabolite of **8**; no **22** was observed.

Kinetic Isotope Effect on Inactivation of iNOS by **8 and **26**.** Inactivation of iNOS with (S)-2-amino-5-(propanimidamido-2,2,3,3,3-*d*₅)pentanoic acid (**26**) showed no kinetic isotope effect on $^Hk_{inact}/^Dk_{inact}$ (1.025 ± 0.071) or on $^H(k_{inact}/K_i)/^D(k_{inact}/K_i)$ (1.159 ± 0.108) (Table 3).

Detection of Heme and Biliverdin from Inactivation of iNOS by **8, **9**, and **11**.** L-NIO and 1400W both convert the active site heme in iNOS to biliverdin IX α , determined by LC–electrospray mass spectrometry and HPLC analysis.²⁶ No

apparent heme loss or biliverdin formation was observed from incubation of iNOS with **8**, **9**, or **11**.

LT-PAGE Assay. To determine whether **8**, **9**, and **11** inactivate iNOS by disruption of the homodimer structure to monomers, iNOS was incubated with L-NIO (**4**), **8**, **9**, and **11**, and low-temperature SDS–polyacrylamide gel electrophoresis (LT-PAGE) was performed. The gels were stained with SYPRO tangerine protein gel stain and imaged for protein quantitation in a Typhoon 9400 imager with a blue laser beam at a wavelength of 488 nm. It was reported that the addition of 1 μM L-Arg and 0.2 mM H₄B produced a stable and noncovalently linked iNOS dimer;⁴⁶ both the iNOS monomer and dimer were present on the gel. Less dimer was detected after incubation with L-NIO (**4**), **8**, **9**, and **11** (Figure 2). The amounts of dimer and monomer remaining after inactivation are listed in Table 4.

DISCUSSION

On the basis of no nitric oxide having been produced and no products detected by LC–MS from incubation of iNOS with up to 2 mM **7–13**, we can conclude that these compounds do not act as substrates, unlike L-NIO (**4**).

Compounds **7–13** all inhibited iNOS activity; all are competitive reversible inhibitors except **12**, which is a noncompetitive reversible inhibitor, and **8**, **9**, and **11**, which are irreversible inhibitors. The inhibitor potency decreases as the amidine alkyl group becomes larger; **7**, which has the smallest sized R group (H), was the most potent inhibitor of iNOS, with a K_i of 0.18 μM . This implies that the active site pocket of iNOS has sufficient room to fit a bulky group at the amidine terminus, but the larger the substituent, the greater the steric hindrance with surrounding residues. The only reversal in apparent size is **10** and **11**, with the K_i for the isobutyl analogue (**11**) being half of that for the isopropyl analogue (**10**).

Because crystal structures of these L-NIO analogues bound to iNOS were not available, computer models of **10** and **11** were manually built according to the binding model of substrate (L-Arg) and docked into the murine iNOS structure (IN2N).⁴⁷ The docked models were then energy-minimized and subjected to molecular dynamics simulations. The models right after the initial minimization are shown in Figure 3 and those of the final frame of the simulation in Figure 4. Both **10** and **11** remain stably bound throughout the 1 ns MD simulation. The side chain isopropyl and isobutyl groups of **10** and **11**, respectively, are tucked into a hydrophobic pocket lined by Pro344, Val346, and Phe363.

In the computer model of **10** after minimization (Figure 3A), tight contacts are shown between the isopropyl group and the Pro344 carbonyl as well as the heme iron, whereas **11** does not have those tight contacts (Figure 3B). MD calculations relax all of the tight contacts but at the cost of distortion of the ligand's geometry, especially around the amino acid moiety, which departs from what is known about how L-Arg binds (Figure 4). Although just computer models, it is interesting that after minimization they are more agreeable with the K_i measure-

Table 2. Kinetic Constants for **4**, **8**, **9**, and **11**

	L-NIO (4)	8	9	11
k_{inact} (min ⁻¹)	0.073 \pm 0.003	0.0743 \pm 0.0073	0.131 \pm 0.0095	0.347 \pm 0.006
K_i (μM)	13.7 \pm 1.6	243 \pm 31.4	674 \pm 80	4009 \pm 426
k_{inact}/K_i (μM^{-1} min ⁻¹)	0.0053 \pm 0.0007	0.00031 \pm 0.000028	0.00020 \pm 0.0000096	0.000087 \pm 0.0000011

Table 3. Kinetic Isotope Effect on iNOS Inactivation by 8 and 26

	8	26	H/D
k_{inact} (min^{-1})	0.0743 ± 0.0073	0.073 ± 0.0063	1.025 ± 0.071
K_i (μM)	243 ± 31.4	278 ± 27.7	0.884 ± 0.068
k_{inact}/K_i ($\mu\text{M}^{-1} \text{min}^{-1}$)	0.00031 ± 0.000028	0.000265 ± 0.000015	1.159 ± 0.108

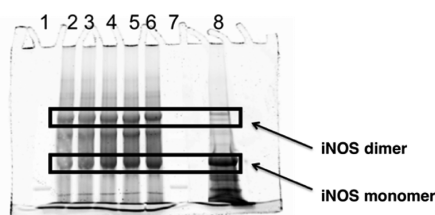


Figure 2. LT-PAGE gel of iNOS with L-NIO (4), 8, 9, and 11: lane 2, native iNOS; lane 3, 4; lane 4, 8; lane 5, 9; lane 6, 11; lane 8, denatured iNOS.

Table 4. Amount of Dimer Remaining after Inactivation

lane		% iNOS dimer
2	native iNOS	65 ± 5
3	4 with iNOS	51 ± 4
4	8 with iNOS	46 ± 2
5	9 with iNOS	47 ± 5
6	11 with iNOS	49 ± 5
8	denatured iNOS	<2

ments, where 10 has a binding affinity poorer than that of 11. This may result from steric crowding of 10; 11 has a longer, and therefore more flexible, isobutyl tail, which can prevent unfavorable steric clashes and instead make more favorable nonbonded contacts (Figure 3).

Over the course of the simulation, the distance between the center of mass of the alkyl groups and iron is $4.49 \pm 0.30 \text{ \AA}$ for 10 and $3.90 \pm 0.17 \text{ \AA}$ for 11, with the alkyl groups remaining positioned directly over the iron. The small standard deviation indicates that the alkyl groups are quite stable and remain close to the heme iron. Such a close approach directly over the iron leaves insufficient room for O_2 to bind, which would preclude their substrate activity, as observed. In sharp contrast, L-NIO is isosteric with L-Arg, and the crystal structure of the eNOS–L-

NIO complex (a structure in iNOS is not available)⁴⁸ shows that there is sufficient room for O_2 to bind to the heme iron. Moreover, the comparable size of NH_2 and CH_3 of the acetamido of L-NIO allows either the protonated imino group to be available to donate a proton to the heme peroxide and act as a substrate or the methyl group to block proton donation and lead to inactivation, as observed. In contrast, the L-NIO analogues with a larger alkyl group cannot rotate, because of the limited space in the NOS active site (Figure 3), to bring the imino group closer to the iron. Therefore, blockage of binding of O_2 to heme and the lack of a close proton donor are the reasons why compounds 8–13 do not act as substrates. The only exception is 7 ($\text{R} = \text{H}$), which also should allow O_2 to bind to the heme iron because it is smaller than L-NIO and should be able to rotate, but 7 does not act as a substrate or as an inactivator. Although the hydrogen of the formamido group of 7 can presumably rotate toward the heme, unlike L-NIO, whose methyl group can fill the void left by the rotated imino group, the hydrogen of 7 cannot, and the protonated imino group of 7 is not aligned properly to protonate the heme peroxide. Therefore, 7 does not act as a substrate or inactivator, as observed.

Compounds 8, 9, and 11 exhibit weak irreversible inhibition of iNOS at concentrations much higher than that of L-NIO (4). Although K_i increases with an increase in size, k_{inact} also increases; however, the overall efficiency (k_{inact}/K_i) diminishes with size, and that for L-NIO is 17–61 times greater than those of the other three compounds. No inactivation was observed if the R group of the amidine was branched at the α -carbon atom, replacing one or both of the α -protons, as in 10 and 12. Compound 13 ($\text{R} = \text{Bn}$) also has a secondary carbon at that position, but it is not an irreversible inhibitor, probably because its binding is so weak ($K_i = 2.4 \text{ mM}$).

Fast et al. reported that L-NIO is both an inactivator and a substrate of iNOS;²³ incubation of iNOS with L-NIO gives N^w -hydroxy-L-NIO, which inactivates iNOS, although it is not on

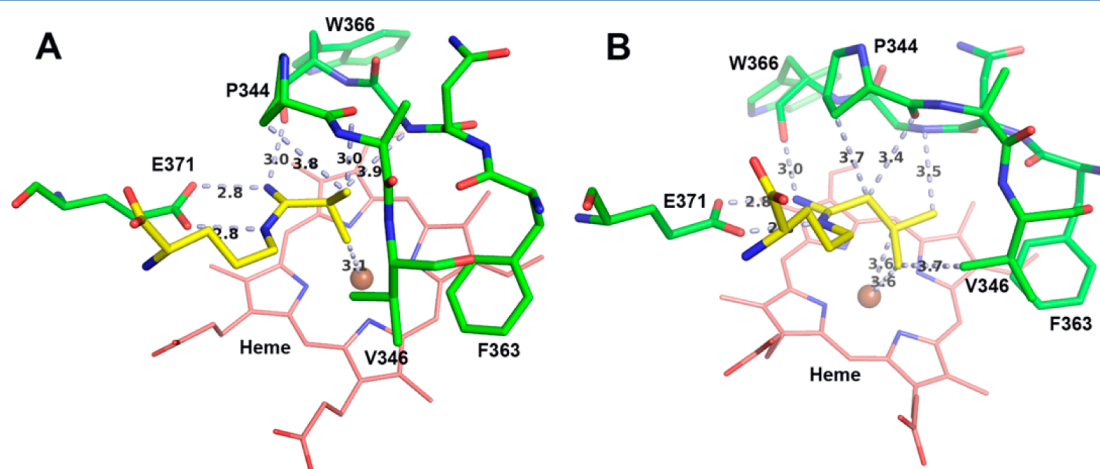


Figure 3. Models of (A) 10 and (B) 11 right after the initial minimization. Major distances of $<4.0 \text{ \AA}$ are marked in angstroms, including the three H-bonds that anchor the inhibitor to the NOS active site glutamate. This figure was prepared with PyMol (www.pymol.org).

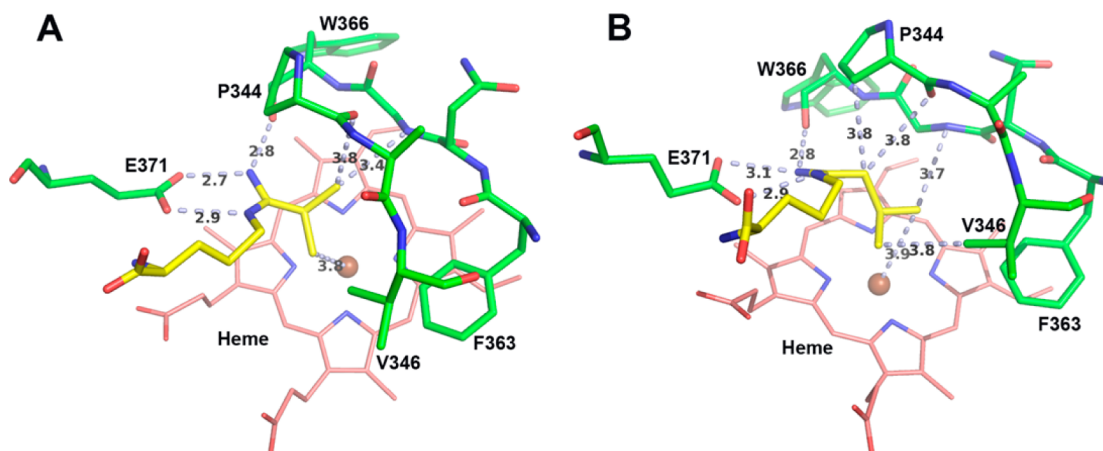


Figure 4. Models of (A) **10** and (B) **11** in the final frame of the simulation. Major distances of <4.0 Å are marked in angstroms, including the three H-bonds that anchor the inhibitor to the NOS active site glutamate. This figure was prepared with PyMol (www.pymol.org).

the inactivation pathway from L-NIO. Compound **22** was designed as the potential hydroxylation product of **8**, but no **22** was observed, again supporting the lack of detection of nitric oxide from incubation of **8** with iNOS. Furthermore, **22** did not inactivate iNOS.

Compound **8** is a time-, concentration-, and NADPH-dependent inhibitor of iNOS. To determine if inactivation proceeds by abstraction of a hydrogen atom from the amidino ethyl group, (*S*)-2-amino-5-(propanimidamido-2,2,3,3,3-*d*₅)-pentanoic acid (**26**) was synthesized. Although it inactivated iNOS, it did so with no kinetic deuterium isotope effect on k_{inact} or $k_{\text{inact}}/K_{\text{I}}$ (Table 3), consistent with an inactivation mechanism that does not involve amidine modification.

One other possible inactivation mechanism was tested: iNOS dimer disruption. The results, demonstrated by low-temperature PAGE in the presence of H₄B and L-arginine (Figure 2 and Table 4), indicated that of the 65% SDS-resistant iNOS dimer that appeared in the control, only 16–19% was converted to iNOS monomer after inactivation with compounds L-NIO, **8**, **9**, and **11**. However, this does not account for complete loss of enzyme activity, although it could be one of the mechanisms of inactivation.

L-NIO²⁵ and 1400W²⁶ appear to inactivate iNOS by conversion of their heme cofactor to biliverdin, which requires molecular oxygen. With **8**, **9**, and **11**, which our computer model suggests prohibit oxygen binding, no biliverdin could be detected by HPLC with either UV or MS detection. Also, $<5\%$ of heme was lost by incubation of iNOS with **8**, **9**, or **11**. Therefore, the mechanism of inactivation of iNOS by these compounds cannot yet be determined but is definitely different from that by L-NIO,²⁵ where substrate turnover via *N*-hydroxylation occurs, and methyl amidines L-NIO²⁵ and 1400W,²⁶ in which subsequent heme degradation was previously described.

CONCLUSIONS

A series of L-NIO analogues was synthesized to determine if the dual substrate/inactivation properties of L-NIO occurred throughout the series and if the size of the alkyl group determined the ratio of turnover to inactivation, as was observed for L-NIO²⁵ and 1400W.²⁶ None of the analogues acted as substrates for iNOS. All were inhibitors, with inhibitory activity diminishing with increased size of the amidine substituent, but only analogues **8** (R = Et), **9** (R = *n*-Pr),

and **11** (R = *i*-Bu) produced irreversible inhibition. Although **7** (R = H) was the most potent inhibitor, even more potent than L-NIO, it did not inactivate iNOS. One common feature of the compounds that produced irreversible inhibition was the primary or secondary carbon adjacent to the imino carbon of the amidine group. Some iNOS dimer was lost (16–19%) with inactivation by **8**, **9**, and **11**, but that was not much different from the amount that was lost by L-NIO inactivation. However, unlike L-NIO, no nitric oxide was formed, no *N*-hydroxylated product was formed, no heme was lost, and no biliverdin was formed upon inactivation of iNOS by **8**, **9**, and **11**. Therefore, the mechanism of inactivation of iNOS by **8**, **9**, and **11** is different from that with L-NIO and 1400W, but that mechanism is still unknown.

ASSOCIATED CONTENT

Supporting Information

Kinetic data for **8**, **9**, **11**, and **26**; HPLC of control, **4**, **8**, **9**, and **11** with iNOS; preparation and characterization of compounds; and ¹H NMR and ¹³C NMR spectra of inhibitors. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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